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Spermatogenesis and Sperm Storage in the Testes of the Behaviorally Distinctive Male Morphotypes of *Macrobrachium rosenbergii* (Decapoda, Palaemonidae)

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Abstract. Adult males of the freshwater prawn *Macrobrachium rosenbergii* differentiate into three morphotypes. Each morphotype develops in sequence in the adult male population from small males through orange claw males to dominant blue claw males. Small males and blue claw males are sexually active, while orange claw males represent a sexually inactive intermediate stage engaged in somatic growth.

To further examine these behavioral characteristics on a physiological level, testes from each of the morphotypes were cultured *in vitro*. The rate of [^{3}H]-thymidine incorporation, representing DNA synthesis, and the amount of sperm released into the culture media were recorded. In parallel, the relative wet weight of testes from the different morphotypes were recorded and histological preparations were examined.

The relative weight of the testes from small males was significantly greater than in the other morphotypes. The testes of small males were both active in spermatogenesis and contained a large amount of mature sperm. Transition to the orange claw morphotype is marked by spermatogenically active testes, characterized by a multilayered spermatogenic zone and a high rate of thymidine incorporation. The testes of the orange claw morphotype develop into organs containing mainly spermatocytes. The fully differentiated orange claw male will transform by a metamorphic molt into the sexually active blue claw male. Almost no spermatogenic activity was recorded in the testes of the blue claw males. The testes of the blue claw males contained, almost exclusively, mature sperm.

The anatomical, physiological, and histological status of the male morphotypes are closely associated with the growth pattern and behavioral attributes of the morphotypes as they differentiate through the developmental pathway from the small male through the orange claw to the blue claw male morphotype.

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Introduction

Among mature males of the freshwater prawn, *Macrobrachium rosenbergii*, three distinctive morphological types appear: small males, orange claw males, and blue claw males (Ra'anan, 1982). The male morphotypes differ from each other in their claw color, relative claw length, and spination (Kuris *et al.*, 1987) as well as in their reproductive behavior (Ra'anan and Sagi, 1985) and growth rates (Ra'anan and Cohen, 1985). Two of the morphotypes—the blue claw and small males—represent two alternative mating strategies. Large blue claw males are able to court actively and protect females prior to and during mating. Small males engage in a form of 'sneak copulation' consistent with their small size and high mobility. Orange claw males are a rapidly growing intermediate phase from the small male to the blue claw morphotypes. Orange claw males infrequently engage in reproductive activity (Ra'anan and Sagi, 1985).

The male reproductive system in *Macrobrachium rosenbergii* consists of a pair of testes, a pair of vasa deferentia, and a pair of genital pores. The testes are elongated structures, united at their anterior end, which lie between the dorsal surface of the hepatopancreas and the heart. Each testis consists of a large number of lobules or cylinders compactly held together by connective tissue (Pat-

wardham, 1937; Sreekumar *et al.*, 1982). Spermatogenic cells and sustentacular cells constitute the two major types of cells within the testicular cylinder. Mature spermatoza are confined to a lumen within the testicular cylinder, described as a blind-ending cyst by Dougherty and Sandifer (1984).

Previous work showed that the relative weight and anatomical development of the reproductive system correlate with the morphological and behavioral changes in males occurring during the morphotypic differentiation process (Sagi and Ra'anan, 1988). Since the three male morphotypes are reproductively distinct, it was of interest to examine the difference in their gonadal structure and activity. The present study focuses on differences among male morphotypes in the testes *i.e.*, gonado-somatic index and gonad activity, as observed *in vitro* by sperm release and [^{3}H]-thymidine incorporation into DNA in the testes. In addition, the histological structure of the testes from different male morphotypes was studied. We found that the structural, physiological, and biochemical properties of the testes are closely associated with the morphological and reproductive states of these morphotypes.

Materials and Methods

Selection of prawns

Male prawns were selected from a single aged population after 150 days of growth in earthen ponds at the Dor Aquaculture Research Station, Israel. Prawns were divided into five categories according to coloration, second pereiopod (claws) shape and spination, and propodus and carapace length (Ra'anan, 1982; Ra'anan and Cohen, 1985; Kuris *et al.*, 1987). Carapace and propodus length were measured with a caliper to 0.1 mm. Carapace length was defined as the distance from the posterior margin of the right orbit to the midline of the posterior margin of the carapace. The propodus, with the joint flexed, was measured along the lateral face from the proximal lateral condyle to the distal tip.

Fifteen prawns of each category were selected for study. Small males, weak orange claw, strong orange claw, and blue claw forms were selected according to the methods described by Kuris *et al.* (1987). Pretransforming strong orange claw males were selected according to the characteristics described by Sagi and Ra'anan (1987).

Gonado-somatic Index (GSI)

The weight of the prawns and the wet weight of the gonads were measured, after drying them thoroughly on filter paper, using a precision balance (±1 mg). The GSI was calculated as follows:

$$\text{GSI} = 100 \times \frac{\text{gonad wt.}}{\text{body wt.}}.$$

Organ culture ([^{3}H]-thymidine incorporation and sperm release)

The animals were dissected under sterile conditions upon arrival in the laboratory. The testes were clipped off the sperm duct and were incubated intact in 2 ml culture medium (12 well, cell culture plates, Nunc). The medium consisted of Dulbecco's Modified Eagles Medium (DMEM) containing 1 m*M* glutamine and 3.2 g $NaHCO_3$ which was adjusted to *M. rosenbergii* osmolarity by supplementing the following salts: KCl (160 mg/l), $MgCl_2 \cdot 6H_2O$ (240 mg/l) and NaCl (3.16 g/l). The salts were added according to the concentrations found in hemolymph of *Macrobrachium* (Stern, 1985) and pH was adjusted to 7.6 with 1 *N* NaOH (Nagamine *et al.*, 1980). Radiolabelled [^{3}H]-thymidine was added as a tracer at zero time (amount: 2 μCi/ml, specific activity 52 Ci/mmol, New England Nuclear). After 24 hours of incubation at 25°C, the organs were removed from the culture dishes. Morphological and histological examinations, performed on some of the cultured organs at this stage, revealed no apparent signs of deterioration. Sperm remaining in the medium (probably released via the excised sperm duct opening) were counted using a hemocytometer. The tissue was homogenized at 1–2°C in 2 ml Tris HCl buffer 10 m*M*, pH = 7.4. The homogenate of the testes was incubated for an additional 5 min on ice after the addition of 2 ml 10% TCA solution and then filtered through fiberglass filters (GFC, Whatman). The papers were then rinsed once with cold 10% TCA solution and twice with 98% ethyl alcohol. The paper was dried then incubated in scintillation fluid (toluene:triton, 1:3, overnight at 25°C). Radioactivity was measured in a liquid scintillation spectrophotometer. Results are expressed in counts per minute per 10 milligram wet weight of testes.

Histological observations

From each morphotype, testicular samples from five individual prawns displaying unequivocal morphotypic characteristics were selected for histological study. The prawns were dissected and the testes were removed and fixed in Brodski fluid (Brodski, 1960). The samples were then embedded in paraffin, serially sectioned at 7 μm, and stained with Ehrlich's hematoxylin-eosin (Humason, 1967).

Statistical analysis

Analysis of variance (Sokal and Rohlf, 1981) was performed to analyze variation in: (a) the Gonado-somatic

Table I

The Gonado-somatic Index of different Macrobrachium rosenbergii *male morphotypes*

Morphotype	GSI ± SE
Small male	0.24 ± 0.04
Weak orange claw	0.10 ± 0.03
Strong orange claw	0.09 ± 0.01
Pretransforming orange claw	0.16 ± 0.03
Blue claw	0.14 ± 0.01

Index, (b) [^{3}H]-thymidine incorporated in counts per minute per 10 mg gonads, and (c) the amount of sperm released into the culture media per 10 mg gonads among the various male morphotypes. The SPSS (Statistical Package for the Social Sciences) was used for this analysis.

Results

Gonado-somatic Index

Table I shows the GSI, calculated as a percentage of total body weight. It is apparent that strong orange claw males are characterized by a significantly lower Gonado-somatic Index when compared to small males and blue claw males, 0.09 ± 0.01 *versus* 0.24 ± 0.04 ($P < 0.005$) and 0.14 ± 0.01 ($P < 0.05$), respectively. Among the orange claw males, the Gonado-somatic Index of the strong orange claw males does not differ significantly from the weak orange claw males, while it is significantly lower than that of the pretransforming strong orange claw males ($P < 0.05$).

[^{3}H]-*Thymidine incorporation and sperm release by the gonad* in vitro

The incorporation of [^{3}H]-thymidine into cultured cells measures the rate of DNA synthesis (Collins, 1977). Our preliminary studies showed that 70%–85% of [^{3}H]-thymidine incorporated into *M. rosenbergii* testes in organ culture was indeed found in the cellular DNA fraction. This was shown by inhibition of thymidine incorporation by hydroxyurea and by the lability of the [^{3}H]-thymidine labelled macromolecules towards DNA digestion. Therefore we investigated the differences among the male morphotypes in their ability to synthesize DNA in organ culture.

Radiolabelled thymidine incorporation into the gonad during a 24-hour period and the amount of sperm released by the gonad into the culture media were determined (Fig. 1). For strong orange claw males, high levels of incorporation were associated with a low amount of sperm released by the gonad: 2970 cpm/10 mg tissue and 1.2×10^5 sperm/10 mg, respectively. On the other hand, high amounts of sperm were released, although very low levels of thymidine incorporation were detected in gonads of blue claw males (2.6×10^6 sperm/10 mg and 317 cpm/10 mg). The differences in sperm release and in the levels of [^{3}H]-thymidine incorporation between strong orange claw and blue claw males are statistically significant ($P < 0.005$ and $P < 0.05$, respectively).

Gonads removed from small males were characterized by a relatively high level of [^{3}H]-thymidine incorporation (4374 cpm/10 mg). This level of incorporation was significantly higher than that of blue claw males ($P < 0.01$), but not significantly different from the high levels of incorporation for strong orange claw and weak orange claw males. Gonads of small males released a relatively high amount of sperm into the media (1.5×10^6 sperm/10 mg). This was significantly higher than sperm released from strong orange claw and pretransforming strong orange claw male testes ($P < 0.01$), but not significantly different from the amounts released by blue claw males (Fig. 1).

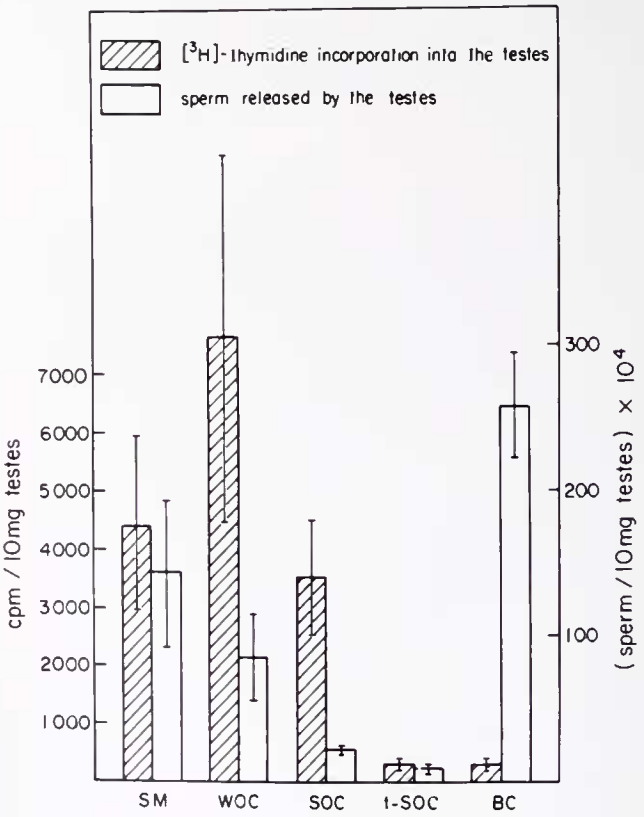

Figure 1. Sperm release and [^{3}H]-thymidine incorporation in organ culture of testes of *Macrobrachium rosenbergii* male morphotypes. The data represent means of 15 experiments and the variation is expressed as SE. SM = small male, WOC = weak orange claw, SOC = strong orange claw, t-SOC = pretransforming strong orange claw, BC = blue claw.

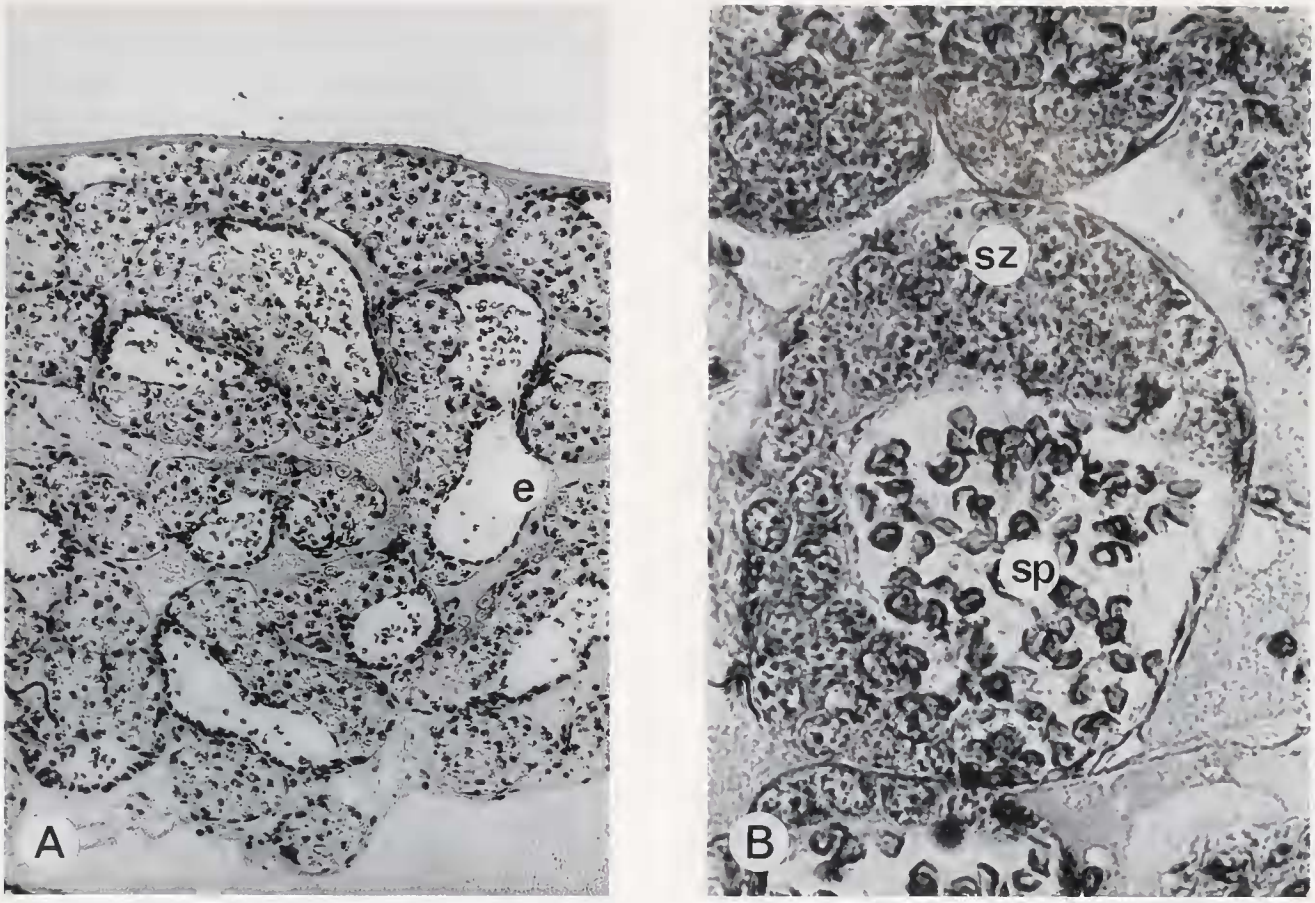

Figure 2. Light photomicrography of a cross-section through one testicular lobe removed from a small male: A, general cross-section (×50), B, a typical testicular cylinder (×200). e—epithelium, sz—spermatogenic zone, sp—mature spermatozoa.

The gonads removed from the pretransforming strong orange claw males were characterized by low levels of [^{3}H]-thymidine incorporation (331 cpm/10 mg). This level of incorporation is lower than all forms of males ($P < 0.01$) except blue claw males. The inactive testes of the pretransforming orange claw males were associated with a low amount of sperm released into the media, 9×10^4 sperm/10 mg, which was significantly low relative to other male morphotypes ($P < 0.01$), but not significantly different from the strong orange claw males.

Histological observations

The testicular lobes are composed of long cylinders compactly held together by connective tissue. Light microscopy revealed differences in the content of the cylinders among the different male morphotypes.

The testes of small males and weak orange claw males contained cylinders, most of which were enveloped by a single layer of epithelium (Fig. 2A, e). Part of the epithelium was multilayered and included cells of variable size, forming a spermatogenic zone (Fig. 2B, sz) containing germinal cells and sustentacular cells. Mature spermatozoa were seen in the lumen of each cylinder (Fig. 2B, sp).

The spermatogenic zone in the testes removed from strong orange claw and pretransforming strong orange claw males was thinner, occupying less space than in the testes of small males and weak orange claw males (Fig. 3A, sz). The cylinders contained spermatocytes (Fig. 3A, sc) which appeared similar in size, shape, and cytological features. While each cylinder contained spermatocytes of a uniform appearance, this appearance may vary from cylinder to cylinder (Fig. 3B, 1 and 2), probably corresponding to spermatogenic stage.

The testicular cylinder of blue claw males contained mature spermatozoa almost to the exclusion of other cell types (Fig. 4, sp). The spermatogenic zone was bearly observable (Fig. 4B, sz).

In all cases, the histological evidence for sperm accumulation in the dissected testes corresponded to the

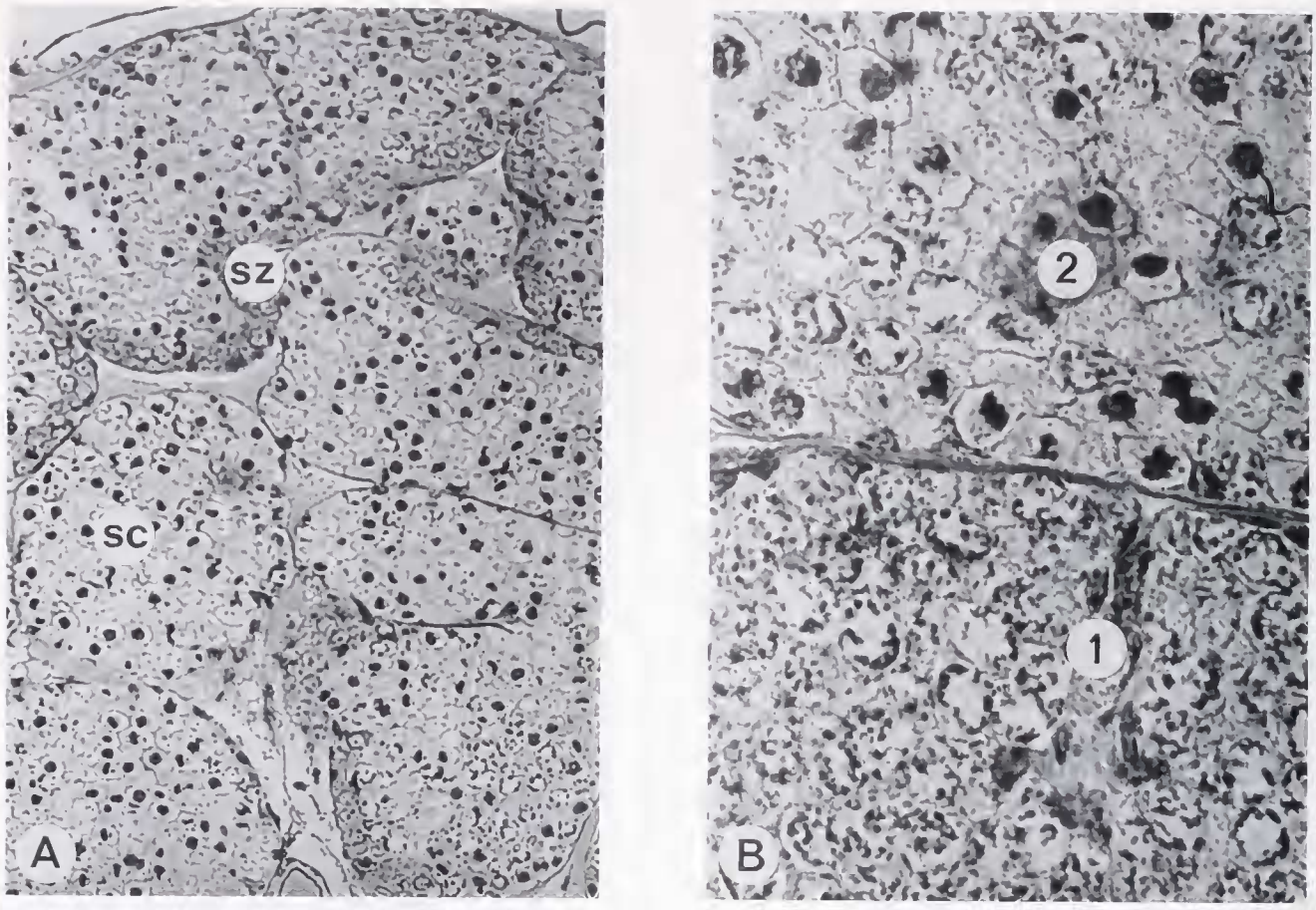

Figure 3. Light photomicrography of a cross-section through a testicular lobe removed from a strong orange claw male: A. general cross-section (×100), B. two neighboring testicular cylinders (×200) differing from each other in spermatogenic stage (1, 2). sz—spermatogenic zone, sc—spermatocytes.

amount of mature sperm released into the culture media after 24 hours of incubation.

Discussion

When polymorphism occurs among mature males in a population, different male morphotypes may adopt alternative mating strategies. This has been reported for insects (Alcock *et al.*, 1977; Ward, 1983), freshwater fishes (Keenleyside, 1972; Constantz, 1975; Gross and Charnov, 1980), some terrestrial mammals (Gadgil, 1972), and in crustaceans (Ra'anan and Sagi, 1985; Shuster, 1987). In most cases, individuals practice only a single reproductive strategy throughout their lifetimes. However, in some cases alternative mating patterns are part of a developmental sequence during a single lifetime (Dominey, 1980). The latter mechanism was described for sexually mature *Macrobrachium rosenbergii* males, in which small males may transform through the orange claw phase into the dominant blue claw males (Ra'anan and Sagi, 1985).

Among *M. rosenbergii* mature males, the small males employ a 'sneak copulation' strategy (Teleckey, 1984). Yet they retain the potential for somatic growth. Like small males in some fish species (Warner and Robertson, 1978; Robertson and Warner, 1978; Warner and Lejuene, 1985), the small *M. rosenbergii* male has a relatively large reproductive system (Sagi and Ra'anan, 1988) and relatively heavy gonads. These properties suit the small male mating strategy well, *i.e.*, numerous mating attempts and relatively little reproductive success (Ra'anan and Sagi, 1985). Hence, its testes contain relatively large amounts of mature sperm (Figs. 1, 2) and are actively engaged in spermatogenesis.

When the small male becomes an orange claw male, the balance between reproductive effort and somatic growth shifts. This is shown by an increase in growth rate (Ra'anan, 1982), an increase in the relative weight of the midgut gland, and a reduction in the relative weight of the reproductive system (Sagi and Ra'anan, 1988). As expected, these phenomena are accompanied by a de-

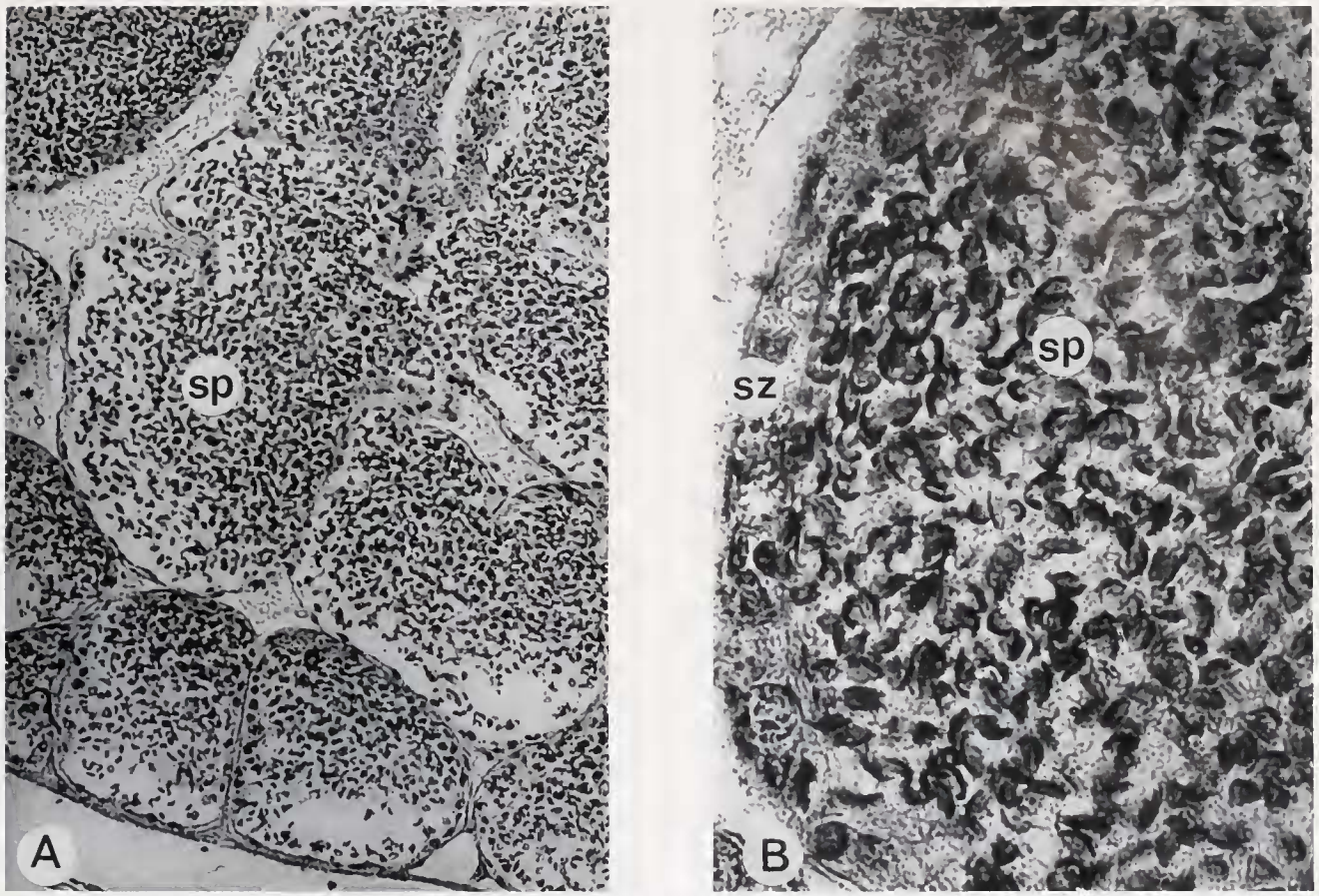

Figure 4. Light photomicrography of a cross-section of a testicular lobe removed from a blue claw male: A. general cross-section (×50), B. a typical testicular cylinder (×200). sp—mature spermatozoa, sz—spermatogenic zone.

crease in the relative weight of the gonad. Two parallel processes occur in the testes during the orange claw phase which indicate the intermediate nature of this morphotype. First, mature sperm, found in large amounts in the small male testes, are in reduced abundance in the weak orange claw males and almost disappear in the later strong and pretransforming orange claw stages. Thus, the orange claw male is almost sexually inactive, growing rapidly to reach dominant male size and appearance. Second, the spermatogenesis rate increases when small males molt to the weak orange claw male phase. Histologically, the [^{3}H]-thymidine incorporation data are supported by the multilayered spermatogenic zones within the testicular cylinders. In more advanced orange claw male phases, the spermatogenic activity decreases and almost ceases prior to metamorphosis into the blue claw male morphotype (Fig. 1). In the strong and pretransforming orange claw males, the spermatogenic zone becomes thinner while testicular cylinders become filled with spermatocytes (Fig. 3). Dougherty and Sandifer (1984) observed two apparently exceptional specimens that appeared to be in synchrony with respect to spermatogenesis. Our results suggest that these were orange claw males. These properties are consistent with the status of the orange claw morphotype as an intermediate stage, leading to transformation into the dominant blue claw morphotype.

The testes of the blue claw male contain only mature sperm, while the spermatogenic zone virtually disappears and spermatogenesis comes to a standstill. Thus, blue claw male testes are used exclusively for sperm storage, a characteristic that complements the dominant reproductive status of the blue claw male (Ra'anan and Sagi, 1985). That the blue claw male uses the stored sperm rather than producing sperm is consistent with the suggested pattern of replacement of blue claw males by transforming orange claw males in *M. rosenbergii* populations. This pattern of growth and metamorphosis has been termed the 'leap frogging' pattern (Ra'anan and Cohen, 1985).

In summary, whereas the small male both produces and stores sperm, the orange claw male primarily pro-

duces spermatocytes and the blue claw male only stores and uses mature sperm. Hence we can postulate that the small male possesses relatively undifferentiated testes, having the potential to develop—when environmental and social situations permit—through the intermediate phase of the orange claw into a dominant blue claw male.

The observed changes in the male gonads, from an active sperm-producing organ into a mature sperm storage organ, call for a more detailed study of the ultrastructural and biochemical changes in the spermatogenic process occurring in intermediate phases, among the distinct morphotypes, which are not addressed in the present study.

The appearance of distinctive male types, differing in reproductive activity and somatic growth, calls for studies on the mechanism of control of morphotypic differentiation. *M. rosenbergii* could be a valuable model for the study of endocrine regulation of growth *versus* maturation in crustaceans. The organ culture system developed here may also be useful in future studies of the effect of endocrine factors on the activity of the reproductive system in male crustaceans.

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